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 <p>Pediatric RESEARCH</p> <p>Automated oxygen control after birth</p>	<p>A study of microRNAs from dried blood spots in newborns after perinatal asphyxia; a simple and feasible biosampling method</p> <p>Vennila Ponnusamy, Olga Kapellou, Ellen Yip, Jane Evanson, Liang-Fong Wong, Adina Michael-Titus, Ping K Yip and Divyen K Shah</p>
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A study of microRNAs from dried blood spots in newborns after perinatal asphyxia; a simple and feasible biosampling method

Running title:

MicroRNAs from neonatal dried blood spot

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Abstract:

Background: The potential of microRNAs (miRNAs) as bedside biomarkers in selecting newborns with hypoxic-ischemic encephalopathy (HIE) for neuroprotection has yet to be explored. Commonly, blood-based biomarker tests use plasma or serum which don't allow evaluation of both intracellular and extracellular changes.

Methods: We describe a technique to extract and compare expression of miRNAs from a single small 6 mm diameter dried blood spot (DBS) stored at room temperature with those from EDTA-blood, plasma and urine. Three miRNAs (RNU6B, let7b and miR-21RNA) were quantified via extraction and quantitative RT-PCR performed from a DBS and compared with levels from EDTA-blood, plasma and urine. Secondly, candidate miRNAs let7b, miR21, miR-29b, miR-124 and miR-155 in DBS were evaluated as potential biomarkers for HIE.

Results: Candidate miRNAs were extractable in all biosamples from newborns, with the highest expression in DBS. There was a good correlation between miRNAs levels in DBS and EDTA-blood at -80°C. No significant difference was observed in the miRNA levels between the favorable and unfavorable outcome groups for babies with HIE.

Conclusions: Dried blood spots may be useful for studying the potential of miRNAs as biomarkers for brain injury.

Introduction:

The lack of more effective methods in selecting babies for neuroprotection and the need for prognostication for outcomes after hypoxic-ischemic encephalopathy (HIE) pose major challenges to clinicians, patient families and the economy. At present there is no effective tissue biomarker at the bedside to allow selection of babies for neuroprotection within the narrow therapeutic window or for early prognostication of outcomes. A single or a panel of biomarkers with high sensitivity and specificity that is reliable and reproducible at the bedside would be extremely valuable in selecting babies for appropriate interventions. Biomarkers allow objective measurement and evaluation of biological and pathological response to therapeutic intervention (1). Biological sample (biosample) based biomarkers hold much promise with many potential advantages; these are bedside tests, extraction is relatively quick, easy and cheap and the technology involved in analysis has developed rapidly with small volumes of tissue required.

In all CNS injuries, whether in adults, children or newborns, biosample-based biomarker studies have been carried out mostly on blood samples as well as on urine or cerebrospinal fluid (CSF). Blood based biomarker studies have commonly been performed on plasma or serum components (2) and have not analyzed whole blood or the cellular components. However, using plasma or serum exclusively is limited by the need for long term cold storage (e.g. -80°C), cellular debris contamination, hemolysis, and analysis confined to only extracellularly released components within the blood.

Blood sampling in newborns can be challenging due to the small size of the infant, with technical difficulty in vascular access and/or obtaining sufficient volume of non-hemolyzed blood. Dried blood spots (DBS), typically obtained from a heel prick and allowing drops of blood to settle onto absorbent paper, have been routinely used in newborn babies in the first week of life for mass screening and early detection of multiple diseases including cystic fibrosis, congenital hypothyroidism and inborn errors of metabolism (3). The use of DBS to assay biomarkers of brain injury in newborns has yet to be fully explored. Protein biomarkers such as inflammatory molecules, cell structures or trophic factors have been explored in newborns with HIE, but their effectiveness as successful biomarkers in the clinical setting remains to be determined (2, 4). In this study, we report our method of extracting microRNAs (miRNAs) from DBS stored at room temperature.

MiRNAs are endogenous short non-coding single-stranded RNA molecules made up of 18 - 22 nucleotides, which are important in the regulation of gene expression post-transcription (5). They appear to function by binding to the 3'-untranslated region of messenger RNAs (mRNA) from target protein-coding genes leading to gene silencing by mRNA cleavage, translational repression and deadenylation (6). They have been found to influence varying stages of neurogenesis, including cell differentiation, proliferation and synaptogenesis from in vitro and in vivo studies (7). Studies of miRNAs in mice and humans have shown that approximately 70% of identified miRNAs are expressed in brain tissue, in relation to the complexity of the nervous system and its connections (8-10).

However, the physiological and pathophysiological role of miRNA in newborns remains to be fully determined. With organs such as the brain still developing, it is likely that the expression of miRNA in newborns is distinctive, yet related to their counterparts in adults based on their regulatory role. RNU6B belongs to a class of small nucleolar RNA and is frequently used as an “endogenous control” to normalize miRNA expression studies. Let7b and miR-21 have both been shown to be important in adult brain pathology following trauma in animal studies (11-12), ischemia/stroke (13-14), and Alzheimer’s disease (15-16). Recently, miR-29b which is activated during neuronal maturation has been linked to inhibition of neuronal apoptosis (17). Brain specific miRNA 124 has been well noted to influence the nervous system in a number of conditions including nervous system development, neurodegeneration, CNS stress, stroke, neuro-immunity and brain tumors (18). It has been shown to influence neurodevelopment by triggering brain-specific alternative pre-mRNA splicing (19). Additionally, miRNA 124 has been shown to predict neurological outcome following cardiac arrest in adults (20). MiRNA 155 has been shown to be involved in microglia-mediated immune response (21). To date, the role of these miRNAs in newborns remains to be determined.

We set out to prospectively (i) extract sufficient total RNA from a single DBS stored in room temperature from newborn babies admitted to the neonatal unit after suspected hypoxia-ischemia; (ii) quantify the expression of three selected miRNAs (RNU6B, Let7b and miR-21) in DBS compared to those in EDTA-blood, plasma and urine using quantitative RT-PCR and to (iii) explore the role of five selected miRNAs (Let7b, miR-

21, miR-29b, miR-124a and miR-155) normalized to RNU6B (putative endogenous control) as candidate biomarkers for babies with perinatal asphyxia

Results:

Study Population:

Between January and August 2014, 30 newborn babies were recruited from two centers.

From the 30 newborns studied, 19 were selected for therapeutic hypothermia on the basis of standard cooling criteria (22) and 11 infants with perinatal acidosis and no encephalopathy were managed conservatively.

MRI/Clinical Outcome:

Of the 30 babies, 19 had MRI performed between 5 to 35 days (median = 9 days) of life. The pattern of cerebral tissue injury noted on MRI was scored using the system described by Rutherford and colleagues (23). Based on MRI and/or short term clinical outcome at discharge, 23 babies were predicted to have a favorable outcome and seven were predicted to have an unfavorable outcome. The favorable outcome group had 10 babies with normal neurological examination at discharge and no MRI performed, eight babies with normal MRI scans and five babies with minor changes on MRI. The unfavorable outcome group comprised of five babies with severe changes in MRI, one baby who died on second day of life with multi-organ failure associated with severe hypoxia-ischemia, and a baby with severely abnormal neurological examination at discharge and having on-going investigations for a neurologic diagnosis. Overall, 19 babies were treated with therapeutic hypothermia (n=13 in favourable group; n=6 in

unfavorable group). Table 1 describes the baseline characteristics of all babies based on their predicted outcome group.

Total RNA concentration:

Total RNA concentration values (mean \pm SEM) were 11.1 ± 1.6 ng/ μ l for all biosamples and 10.6 ± 2.5 ng/ μ l for DBS alone. The RNA quality values of 260/280 (mean \pm SEM) were 1.68 ± 0.04 and 1.77 ± 0.05 for all and DBS samples, respectively (**Figure 1**) which represent similar quality of RNA from all biofluids.

MiRNA expression:

Three of the miRNAs examined (RNU6B, Let7b and miR-21) were expressed in a majority of the biosamples (plasma, EDTA -blood, urine and DBS) from the babies. In 4 out of 30 plasma and 8 out of 30 urine samples, RNU6B miRNA was not detectable. Correlations were carried out for the expression of RNU6B, Let7b and miR-21 miRNAs in all biofluids with DBS. There was a significant positive correlation between DBS and EDTA-blood for RNU6B, let7b and miR-21(**Figure 2**). There was no statistically significant correlation between the DBS with plasma or urine. The comparison of miRNA expression in different biosamples using mean cyclical threshold (Ct) values from three technical replicates for all three miRNAs analyzed demonstrated significantly lower Ct values (i.e., higher expression) in DBS than in plasma, EDTA-blood and urine in the 3 selected miRNAs studied: RNU6B, Let7b and miR-21 (**Figure 3**). We also observed from the variable times (range 2 to 191 days) in extraction of total RNA from the point of sample collection, it was still possible to extract the selected miRNAs from all samples

after prolonged storage (data not shown). This was true for all biosamples; plasma, EDTA-blood, urine stored at -80 °C and DBS stored in room temperature.

MicroRNAs in HIE:

To study the role of microRNAs in HIE, five neuro-specific miRNAs (Let7b, miR-21, miR-29b, miR-124 and miR-155) were selected. The Ct values were converted to delta Ct, which represents values normalized to a putative endogenous control RNU6B.

There was no significant difference noted between the favorable and the unfavorable outcome groups in the delta Ct values of all candidate miRNAs studied, i.e., Let7b, miR21, miR-29b, miR-124 and miR-155 in DBS (**Figure 4**). It was also noted that delta Ct values were not different between the outcome groups in other biosamples (plasma, EDTA -blood and Urine) for Let7b and miR21.

Discussion:

To our knowledge, this is the first study to demonstrate that it is feasible to extract sufficient quantity and quality miRNAs in newborns from a single 6 mm diameter DBS stored at room temperature and in quantities significantly higher than in other common biosamples, including plasma, urine and blood for analysis of similar total RNA concentration and quality. We also showed that it was possible to extract total RNA and study the expression of miRNA from DBS stored at room temperature for up to 6 months from the time of sample collection. The quantity and quality of RNA, and all candidate miRNAs extracted from DBS were all similar to those from other biofluids after prolonged storage at -80 °C.

RNU6B is a small nucleolar miRNA, and generally low or not expressed in plasma in adults (24). On the contrary, we noted that RNU6B was expressed in most of our plasma samples apart from a small proportion. Presence of RNU6B in most plasma samples could be explained by the hemolysis noted in a significant proportion of our plasma samples (24). Furthermore, it has been noted that there is an increase in nucleated red blood cells in babies in HIE (25). We also noted RNU6B in most of our urine samples, and this may be due to cells in the urine, as uncentrifuged urine was used in this study.

There was positive correlation between EDTA-blood and DBS for all miRNAs examined. There were significantly lower Ct values for RNU6B, let7b and miR-21 observed in DBS compared to plasma, blood and urine, implying a higher expression of miRNAs in DBS compared to other biosamples. This may be related to the DBS representing both intra and extracellular components of blood. There has recently been much interest in tissue biomarkers in the study of CNS pathology in adults (26). DBS have been routinely used in universal screening programs in newborns in the first week after birth for early detection of diseases (3). Recently, Buroker et al (27) have successfully extracted miRNAs from two 1.5 cm diameter DBS which had been stored at -80 °C. Bartha and co-workers (28) studied cytokines and tumor necrosis factor alpha from DBS by immunoaffinity chromatography in babies with neonatal encephalopathy. These samples were stored at -26 °C before analysis. We were able to extract sufficient quantity and quality of miRNAs from a single 6 mm diameter spots from newborns stored at room temperature. One potential reason for our success is that the DBS was

dried completely after collection, and studies indicate that humidity may affect miRNA preservation (29).

In our study, despite our best efforts to ensure that all blood samples were free flowing, hemolysis was noted in some of the EDTA samples, albeit minimally (data not shown). McDonald et al (30) showed that hemolysis increased apparent concentration of some but not all miRNA levels on pre-analytical variables in extraction of miRNA. Additionally, the time from collection of the blood sample to centrifuge, and then storage at -80 °C, was variable due to difficulty in access to the laboratory round-the-clock in our study. It has been shown that delay in centrifuging up to 6 h affected the levels in some candidate miRNAs studied significantly (31). Therefore, our miRNA extraction from plasma could have been affected due to delay in centrifuging some samples. Most studies on miRNAs have used serum or plasma. However, miRNAs can also be released during cell death as apoptotic bodies or bound to proteins like Ago2 and lipoproteins (32). Therefore in pathologies involving cell injury like perinatal asphyxia, it is potentially more valuable to study whole blood in the form of DBS and not just the extracellular components found in serum/plasma. Furthermore, DBS provided highest expression of miRNA given that 10ng of total RNA was equally loaded for all biosamples.

Limitations of our study include the variation in age after birth when the biosamples were obtained from the newborns. This was limited by age when infants were transferred from other hospitals, the time taken for parents to give informed consent and

the availability of research staff to take and process the samples. Our results did not show a variation in the expression levels of the control miRNA in relation to sampling time from birth. It is well recognized that there is a temporal variation in many biomarkers of brain injury in newborns after birth (33). In future studies, serial sampling from each baby to analyze the natural history of these biomarkers associated with varying degrees of brain injury will be carried out. Due to lack of serial testing with the same sample across various time points, it is not possible to comment on the stability of the RNA and miRNAs over time in the DBS and other biosamples from this study. Although the quality values of the total RNA extracted were not within the expected value of 2.0 when analyzed with the NanoDrop spectrophotometer, this was likely due to the circulating RNases present in the biosample which can degrade large RNAs into small RNAs (34). Li and Kowdley have shown that , clinical serum samples contain mainly small RNA molecules, including microRNAs with the absence of large RNA species, such as messenger RNA and ribosomal subunits 18S and 28S when RNA quality was determined using the Agilent 2100 Bioanalyzer (34). Due to the absence of 18S and 28S subunits, it is not possible to perform RNA integrity number for determining the quality of the total RNA in the samples (34). In this study, the mirVana PARIS RNA extraction kit which involves the strategy of combining denaturing reagents and a silica filter was used. This RNA extraction kit has been shown to successfully extract miRNAs with least miRNA degradation and high miRNA quality in comparison to other commercially tested kits (34).

In this small cohort, we were not able to show any difference in the selected candidate miRNA expression in the unfavorable prognosis group compared to the favorable prognosis group.

Larger studies exploring more candidate miRNAs at serial time points in babies presenting with perinatal asphyxia are required to understand the role of specific miRNAs expression in HIE.

Conclusions: We demonstrate that it is feasible to study the expression of miRNA from a very small sample of DBS obtained from newborns. MiRNA expression from DBS correlates well with those from EDTA-blood. DBS is relatively cheap, technically easier to obtain in all age groups, especially in small and sick neonates and simpler for transportation and storage. This also eliminates potential sources of error, common in the pre-analytical stage, associated with blood collection and centrifuging. This novel method using a very small drop of blood as DBS has a potential for use in future studies to explore further the role of miRNAs as possible biomarkers in neonates.

Methods:

Infant recruitment and selection

The study received Research and Ethics approval (REC 13/LO/1738) from National Research Ethics Committee, London and informed written consent was obtained from the parents of recruited participants. Newborn babies were recruited from two tertiary neonatal centers in London; the Royal London Hospital and Homerton University Hospital. Babies were recruited to the study if (i) they were ≥ 36 weeks gestation, fulfilled standard cooling criteria (22) and were selected for mild hypothermia for neuroprotection by the attending clinicians (cooled group) or (ii) infants who were

admitted to the neonatal unit for conservative management with suspected milder hypoxia-ischemia on the basis of metabolic acidosis but did not fulfill the criteria for cooling treatment (non cooled - mild group). Infants with multiple congenital abnormalities or confirmed inborn error of metabolism were excluded.

Biosamples:

The biosamples chosen for determining miRNA expression levels were plasma, EDTA-blood, urine and DBS collected at a median time of 18-19 hours of age in both groups and after the infants in the cooled group had reached their target core temperature (33.5 °C). Of the 1.5 ml of blood obtained from the newborn, one drop was placed on absorbent filter paper (Whatman 903 protein saver card, GE Healthcare life sciences, Little Chalfont, UK) creating a dried blood spot. This was allowed to dry at room temperature and was then stored in a polythene bag with a packet of desiccant to sustain desiccation. The remaining blood was transferred into tubes with sprayed-coated K2EDTA (Fisher Scientific Ltd, Loughborough, UK) and then centrifuged at 15,000 x g for 10 minutes to separate the plasma and blood components (EDTA-blood). The plasma, EDTA-blood, and urine collected from a bag or a catheter sample were all stored at -80 °C until further processed.

RNA extraction

MirVana PARIS isolation kit (Ambion, Life technologies, Paisley, UK) was used to extract total RNA from 50 µl plasma, 50 µl urine, 1 µl of blood sample withdrawn using a 23G needle, and a single 6 mm diameter chad from the center of DBS was collected

using a clean single hole puncher. Total RNA isolation was carried out according to manufacturer's instruction. Briefly, biosamples were suspended in cell disruption buffer to a final volume of 300 µl and vortexed for 10 seconds. The blood in the DBS was resuspended by pipetting up and down using a 1ml pipette tip, then transferring the supernatant to a clean tube. After the samples were mixed with denaturing solution, acid phenol:chloroform mixture was added, vortexed, then centrifuged at 15,000 x g, for 5 minutes at room temperature. The top aqueous layer was carefully collected and mixed with 100% ethanol. The total RNA was collected in a filter cartridge, washed, and eluted using 100 µl of 95 °C pre-heated elution buffer. Using the NanoDrop 1000 spectrophotometer, the concentration and quality of the total RNA in all samples were analyzed and recorded. All RNA samples were stored at -80 °C until further processed.

TaqMan microRNA assay

TaqMan microRNA assays (Life technologies) for RNU6B , let7b, miR-21, miR-29b, miR-124 and miR-155 were used, which involved the reverse transcription of miRNA followed by a singleplex TaqMan assay reaction performed using real-time PCR amplification. Briefly, 10 µg of total RNA was reverse transcribed using the TaqMan MicroRNA Reverse Transcription kit (Applied biosystems, Life technologies) which contained 1x RT buffer, dNTPs with dTTP, RNase inhibitor and MultiScribe Reverse Transcriptase enzyme and reaction was carried out in a thermal Cyclor at 16 °C for 30 minutes, 42 °C for 30 minutes, 85 °C for 5 minutes and 4 °C for holding. The quantitative PCR amplification was performed using the TaqMan Fast Universal PCR Master Mix (2X), no AmpErase UNG (Applied biosystems, Life technologies), TaqMan

small RNA assay and RT reaction product. The reaction samples (20 μ l) were loaded in triplicates into a 96 well plate. Using the Applied Biosystems 7500 Fast Real-time PCR system, the thermal cycling conditions were: hold at 50 °C for 2 minutes, then at 95 °C for 10 minutes, and then 40 cycles of denature at 95 °C for 15 seconds and anneal/extend at 60 °C for one minute. The results were represented as Ct values. Ct values denote the cyclical threshold in polymerase chain reactions, in which lower Ct values indicate higher expression as a result of amplification in earlier cycles due to abundance of the nucleic acid in the sample.

Outcomes

MR images were independently rated by a neuroradiologist (JE) and a neonatologist with expertise (OK). The pattern of MRI injury was classified into two groups using the system described by Rutherford et al., which has prognostic value in infants who have undergone therapeutic hypothermia (23). Infants with an unfavorable outcome had a severe pattern of injury including: reversed or abnormal signal intensity bilaterally on T1 and or T2 weighted sequences in the posterior limb of the internal capsule; multifocal or widespread abnormal signal intensity in the basal ganglia and thalami; or severe widespread white matter lesions including infarction, hemorrhage and long T1 and T2. Infants with MRIs predictive of a favorable outcome had either normal images or less severe patterns of injury that are associated with normal or mildly abnormal neurodevelopmental outcome. Consensus was reached in cases of disagreement. Infants who died prior to MR imaging were classified in the unfavorable outcome group. Infants who were not cooled were classified as having a favorable outcome if they had a

normal neurologic examination at 24 hours age and were discharged home on suck feeds. These infants did not have MR imaging.

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Table 1: Baseline characteristics of all babies according to predicted outcome

Perinatal characteristics	Favorable outcome n = 23	Unfavorable outcome n = 7
Gestational age, (completed weeks + days)	39+6 (37+2 to 42+4)	40 (35+6 to 41+1)
Male sex, n (%)	12 (52%)	7 (100%)
Birth Weight (grams)	3360 (2000 - 4280)	3504 (2200- 3840)
Apgar score at 10 min	7 (0 - 10)	5 (0 -9)
Worst pH within 1 hr	6.9 (6.48 - 7.30)	6.9 (6.86 - 7.32)
Worst base deficit within 1 hr	-16.1 (-6.4 to -35)	-19.3 (-12.3 to -19.5)
Need for respiratory support at 10 min age, n (%)	12 (52%)	6 (86%)
Need for chest compressions, n (%)	5 (21%)	3 (33%)
Antenatal sentinel event present, n (%)	7 (37%)	2 (18%)

Values are median (range) unless indicated otherwise

Figure 1: RNA extraction: quantity and quality in all 4 biosamples. (a) Total RNA extracted from newborns with HIE showed similar average total RNA concentration as measured in ng/μg. (b) Average total RNA quality in plasma, blood, urine and DBS measured as a 260/280 ratio. NS, non-significant ($P>0.05$) using one way ANOVA with Tukey post hoc test. Data are presented as mean \pm s.e.m.

Figure 2: Expression of miRNAs in DBS compared to plasma, blood and urine biosamples. (a-c) Comparison of RNU6B Ct values in DBS to other biosamples. (d-f) Comparison of Let7b Ct values in DBS to other biosamples. (g-i) Comparison of miR-21 Ct values in DBS to other biosamples. Graphs b, e and h demonstrate significant coefficient of determination (R^2) values for blood and DBS in all 3 miRNAs: RNU6B, $R^2 = 0.270$; Let7b, $R^2 = 0.503$; miR-21, $R^2 = 0.202$. * $P<0.05$, ** $P<0.01$, † $P<0.001$ indicate significance of R^2 between biosamples.

Figure 3: Expression of miRNAs in all 4 biosamples.

Expression of (a) RNU6B, (b) Let7b and (c) miR-21 in all 4 biosamples (each performed in technical triplicates). * $P<0.05$, ** $P<0.01$, † $P<0.001$ indicate significance between biosamples; NS, non-significant ($P>0.05$) using one way ANOVA with Tukey post hoc test. Data are presented as mean \pm s.e.m.

Figure 4: Comparison of normalized expression of selected candidate miRNAs in DBS between favourable and unfavorable outcomes. Mean delta Ct values in DBS for various miRNAs are shown in the graphs: (a) Let7b, (b) miR-21, (c) miR-29b, (d) miR-124 and (e) miR-155. The p values did not show any significant difference between the favourable and unfavorable outcome groups in for all miRNAs. NS, non-significant ($P>0.05$) using Student's t-test. Data are presented as mean \pm s.e.m.

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Figure 1

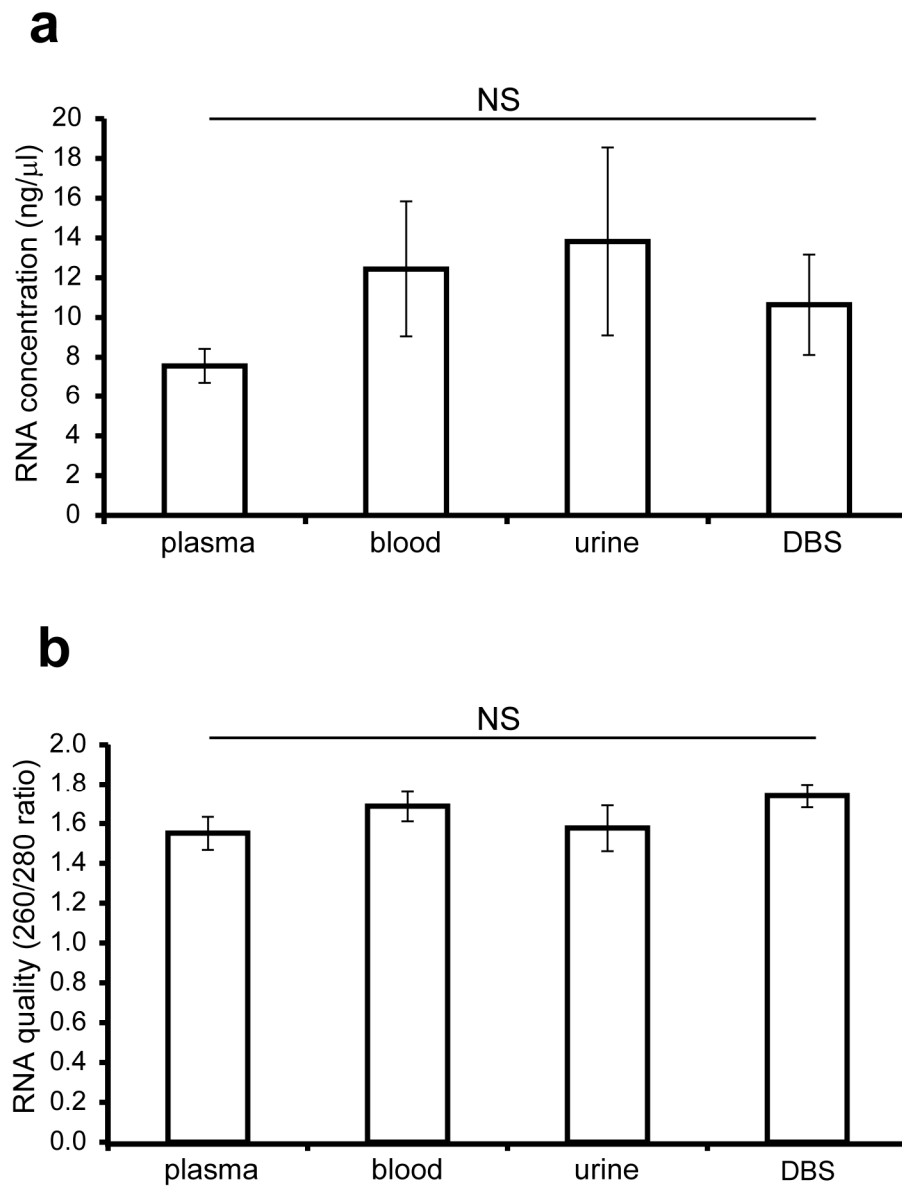


Figure 2

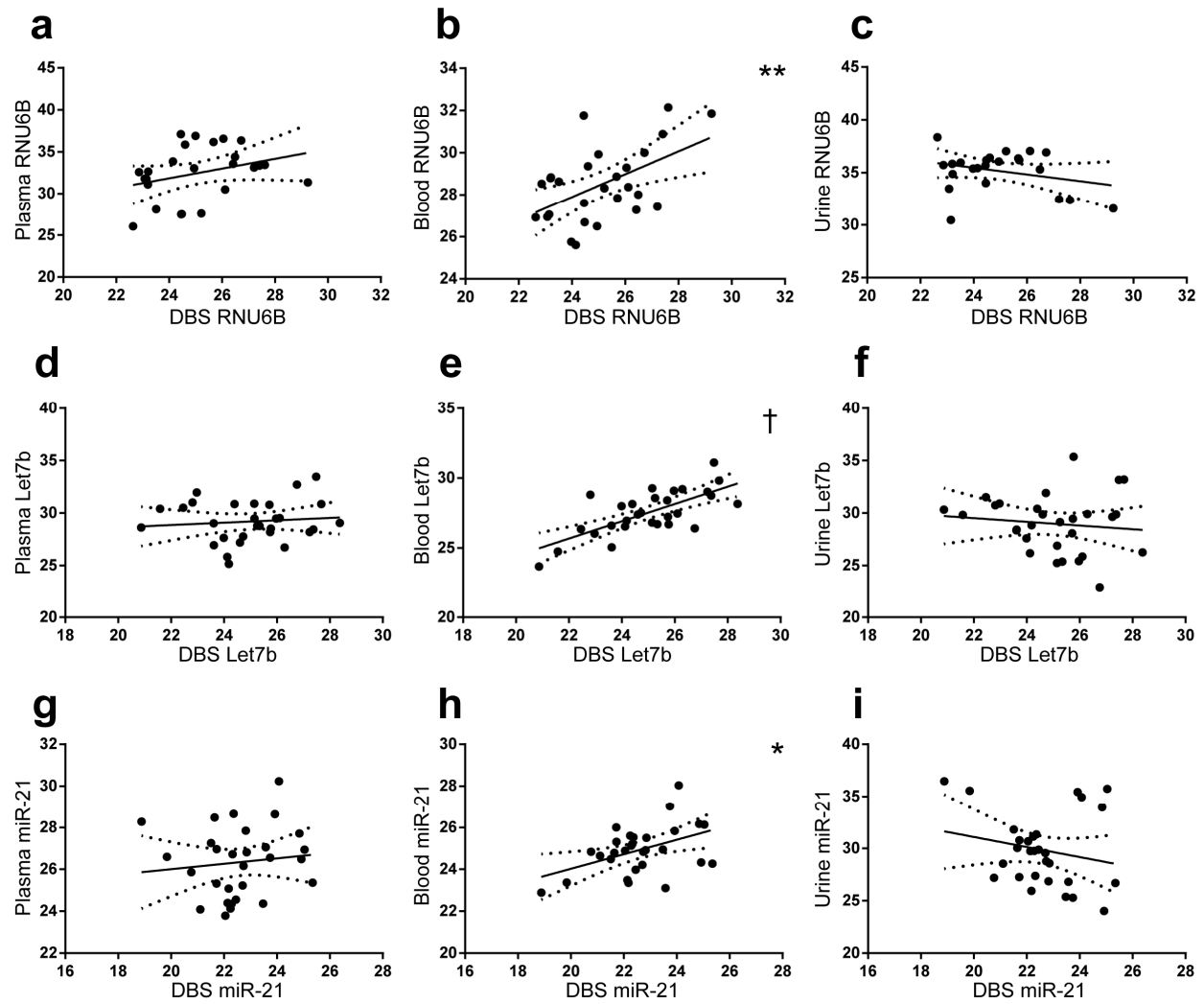


Figure 3

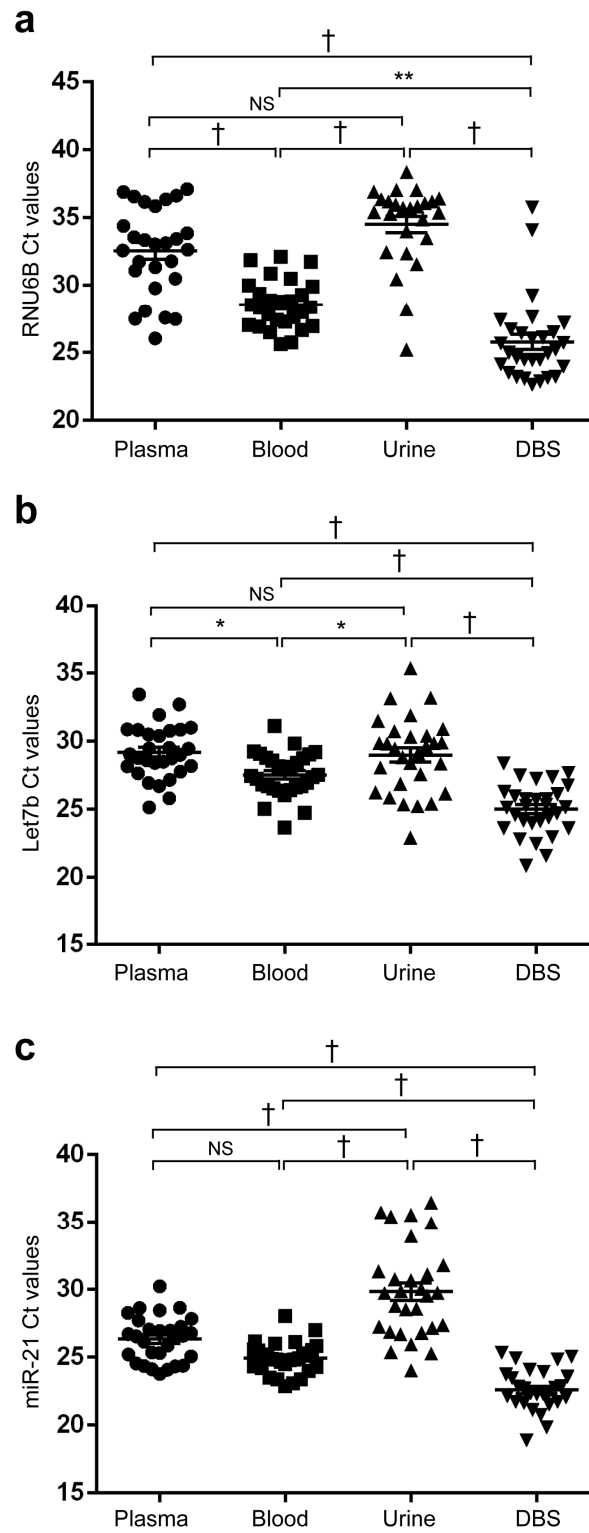


Figure 4

